Application to the Tobacco Industries' Research Committee

for renewal of their support of a Project entitled

THE ENZYMATIC MECHANISM FOR THE DARK FIXATION OF CO_2 BY TOBACCO

from

Department of Biochemistry and Nutrition

University of Southern California

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Scientific Personnel:	Paul D. Saltman, Ph.D. Clyde Stitt, B.S., (Research Assistant) Herbert Spolter, B.S., (Pre-doctoral Fellow)
Duration:	One year
Amount of Grant:	$7,776.00

FOR THE TOBACCO INDUSTRIES' RESEARCH COMMITTEE:

Annual Report and Renewal of Contract

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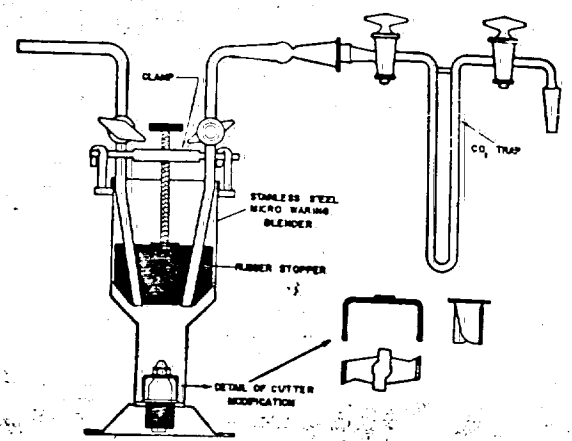
THE YEAR'S PROGRESS: Our problem was to investigate the metabolic processes in tobacco leaves that are operative in the dark fixation of CO_2. Our approach was to identify the first products of the dark fixation by means of paper chromatography. Once these products were known, we planned to proceed with the isolation and study of the enzymatic system that mediates the inter-conversion of the products of the fixation.

An apparatus was designed that would permit the tobacco leaves to be exposed to high concentrations of $C^{14}O_2$ in the dark and, after short periods of exposure to the radioactive material, to be homogenized with 80% ethyl alcohol to stop the reaction. A schematic drawing of the equipment is shown in Figure 1.



CO_2 DARK FIXATION APPARATUS

It consists of a stainless steel micro-Waring Blendor top with slightly modified blades to permit total homogenization of small leaves. The head is fitted with a two-hole stopper machined to give very close fit. The stopper with appropriate ground glass joints is held in position with the clamp as shown. All glass parts are coated with an opaque paint in order to exclude all light.

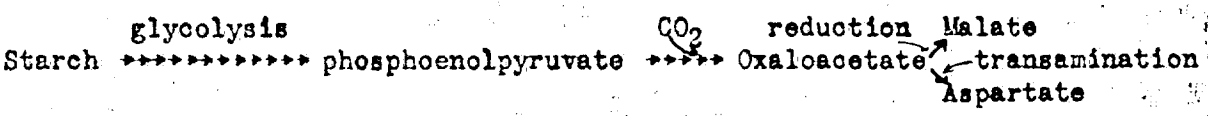
About one gram of young tobacco leaves (footnote 1) are placed around the blade of the blendor to insure rapid and thorough homogenization. The blendor is evacuated and the CO_2, previously generated and collected in a trap, is admitted into the closed system.

At the end of the exposure period, 30 ml. of boiling 80% ethanol is drawn into the reaction chamber by means of an aspirator and the leaves simultaneously homogenized. Exposure periods as short as one minute have resulted in the successful fixation of CO_2 using the above procedure.

The alcoholic extract is centrifuged and stripped under vacuum to a final volume of 2.0 ml. One-tenth of a ml. of the concentrated leaf extract is placed on a large sheet of Whatman No. 1 filter paper along with authentic samples of suspected intermediates. The compounds are resolved by two-dimensional paper chromatography using the solvents 80% phenol - 20% water and butanol - acetic acid - water mixtures. The dried chromatograms are placed in contact with no screen X-ray film to locate the spots that have incorporated the radioactivity. The chromatograms are then sprayed with brom cresol green to locate the organic acids and then sprayed with ninhydrin to locate the amino acids.

(Note:) Seeds of Dixie Bright Tobacco were obtained through the generosity of Dr. Guy Jones, University of North Carolina. Plants were grown by Dr. F. Went at the California Institute of Technology.

During a one-minute exposure of tobacco to $C^{14}O_2$, only two compounds incorporate the label: malic acid and aspartic acid. Significantly, this is the same pattern observed in parallel experiments with succulent leaves. This evidence suggests the following mechanism for the initial fixation:



Attempts to isolate oxaloacetic acid from tobacco as well as from other leaves have been unsuccessful. Communications with Dr. Aranoff and Dr, Thompson who have been concerned with similar experiments, reveal that it is a most difficult if not impossible compound to identify in green leaves. This does not, however, preclude its participation in the reaction above. As another approach to the problem, we have prepared and examined the acetone powders of tobacco leaves for the enzyme mediating the initial fixation, phosphoenolpyruvyl-carboxylase. This enzyme is present in quite high concentration in this tissue.

Exposure of the leaves to $C^{14}O_2$ for periods as long as two hours causes the incorporation of the label into the following compounds: citric, isocitric, malic, fumaric, succinic acids, as well as alanine, aspartic acid, glutamic acid and glutamine. After five minutes exposure, all of the above compounds incorporate the label. These results are most interesting in the light of the similar patterns observed in succulent leaves. The succulents, however, incorporate the major fraction of activity into malic acid, whereas in tobacco leaves, the major fraction seems to be in aspartic acid.

Dark exposed leaves have been allowed to accumulate $C^{14}O_2$, then removed to a light chamber and permitted to photosynthesize. At various intervals of time the photosynthesizing leaves were homogenized and extracted and the compounds identified as indicated above. The most significant observation was that the label disappears from the amino acid fraction quite rapidly but is not incorporated into the sugar fraction as one would expect.

PROPOSAL FOR FUTURE RESEARCH: Our interests are now directed toward the elucidation of the mechanisms operative in the light which cause the loss of radioactivity from the labeled amino acids. It is significant that in the tobacco leaves the major storage fraction for fixed CO_2 in the dark is the amino acid, aspartic acid. Why? Is the transamination reaction from oxalo-acetic acid to aspartic acid of such magnitude to drive the dark fixation toward the amino acid rather than toward the Krebs cycle as found in succulents?

We are concerned that the CO_2 fixed in the dark does not enter into the reversal of glycolysis to yield radio-labeled sugar. It is of interest to understand what is the biochemical role of the fixed carbon dioxide in these green leaves. We would be interested also in determining the effects of various light intensities on the rate of transformation of the amino acids. Is there a key enzyme system which is light sensitive? Or is the phenomenon, rather, the result of a general increase in reducing power within the leaf.

BUDGET:	Salaries:	$5,000.00
	Expendable supplies:	1,200.00
	Permanent equipment:	1,000.00
	Overhead (8%):	576.00
	TOTAL:	$7,776.00

ANTICIPATED DURATION OF WORK: We feel that the major goals of the research program can be attained within the next year. Now that our laboratory is at its full working capacity and the new equipment has been developed and tested, we are certain that even more rapid progress may be made in the future.

FACILITIES AND STAFF AVAILABLE: Through the generosity of the Tobacco Industries Research Committee, our laboratory has now acquired equipment and materials which greatly facilitate our work with radioactive traces. The cooperation of Dr. Fritz Went of the Earhart Plant Research Laboratory at the California Institute of Technology has been placed at our disposal and he has provided us the only means in southern California for the growing of smog-free tobacco leaves.

Our staff has been deprived of the services of Dr. Vicke Haas Lynch who has taken a position at the Carnegie Institute of Biological Research. However, before her departure, she trained Mr. Stitt in the complexities of the art and science of chromatography. Mr. Stitt has become a most proficient and able worker in this laboratory.

Mr. Herbert Stolter is a first year graduate student with a fine academic record both at the University of Southern California and at New York University. We hope that he will be able to make significant contri-

butions through his research.

ADDITIONAL REQUIREMENTS: We are in need of a paper electrophoresis apparatus in order to identify the several unknown compounds appearing on the chromatograms. We are also in need of funds to aid in the purchase of a Beckman DU spectrophotometer to replace the one that has been removed from our laboratory by its original owner. Our group plans to combine its financial resources with two other groups of investigators in the bio-chemistry department to purchase another instrument.

ADDITIONAL INFORMATION: We have been pleased with the response of the students at this university to our increased emphasis in plant bio-chemistry. We hope to intensify this interest and to encourage more students to enter the field. In line with such a program, we have invited Dr. Bernard Axelrod of Purdue University to be a guest lecturer in the bio-chemistry department in the summer of 1956. Dr. Axelrod is one of the foremost authorities in the field of plant bio-chemistry and will give a course in this field. He has also agreed while on this campus to actively participate in the program of CO_2 fixation.